Mass Determination of Intact a-Chain Hemoglobin Adducts to within 0.2 Da Using Mass Peak Profiling from Selected Ion Recording Data with Electrospray Ionization

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1 INTRODUCTION

Assessment of risk to humans and their environment involves identification of chemicals and their concentrations in the environment, how animals are exposed and how the chemicals enter the body, their metabolic disposition or fate, and their toxicology. Observation of intact hemoglobin α- or β-chain adducts from blood can establish that an animal has ingested, inhaled, or absorbed a chemical responsible for the adduct. Mass measurements can establish the presence of adducts. The more accurate and precise the average mass measurement for an adduct, the more compelling is the evidence that a particular adduct is present. Figure 1 illustrates the major known binding sites for human hemoglobin adducts.

Mass accuracies of 0.01% and 0.015% (100 and 150 ppm; 1.5 Da to 2.3 Da for the α-chain) for ions are achievable using MALDI/TOFMS and ESI/QQQMS or ESI/ITMS, respectively (1). *Mass Peak Profiling from Selected Ion Recording Data* (MPPSIRD) utilizing double focusing mass spectrometers has provided error limits of 3 ppm for single measurements using a mass resolution of 20,000 for ions weighing less than 1000 Da (2). Compounds are introduced by GC or from a probe into the mass spectrometer and are ionized in the gas phase by electron impact. Knowledge of ion compositions greatly limits the possible isomers for unidentified analytes and makes feasible literature searches and procurement of standards to conclusively identify compounds (3). However, proteins have much larger masses and are introduced in the liquid phase using electrospray ionization, which lessens sensitivity. This work sought to adapt MPPSIRD to determine the average masses of adducts with greater precision and accuracy.

2 Mass Determination of a Hemoglobin Adduct

Several drops of human blood were added to 1 mL of deionized water and then diluted 9:1 with additional water causing hypotonic lysis. This solution was diluted 1:1 with acetonitrile and made 0.5% in acetic acid to dissociate the hemoglobin, release the heme, and provide the α- and β-chain ions. The resulting solution was filtered and infused into the ESI source attached to a Finnigan MAT 900S double focusing mass spectrometer. Full scanning from m/z 700 to 1500 provided mass spectra that displayed α- and β-chain mass peak profiles with +11 to +20 charge states. Figure 2 is the average of 14 full scans.

Sensitivity was insufficient to use high mass resolution to separate the individual mass peaks that contributed to the composite mass peak observed for hemoglobin chains at 1000 resolution. Only an average mass could be measured. Entrance and exit slits were kept nearly wide open to maximize the signal and provided 1000 resolution. Of the 31 available m/z ratios provided by the data system software, nine monitored each of two adjacent α-chain profiles (used as calibrants) and the remaining 13 m/z ratios monitored the intermediate-mass adduct profile. In Figure 3, the mass range of eight mass increments of 67 ppm delineated the top half of the calibrant profiles. While the hemoglobin solution was infused at 2-6 μL/min, the y-lens polarity in the ion source was reversed for 5 s to induce baseline excursions in the ion chromatograms at all m/z ratios before and after data was acquired for 200 sec. The areas between the baseline excursions were integrated by the data system and plotted to provide the partial profiles in Figures 3b and 3c for the glycate adduct in the +16 and +17 charge states. The average masses of all partial profiles were determined as the weighted average of up to nine points.

Fig. 1

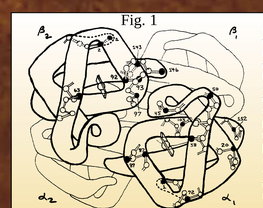

Figure 1. A view of the quarternary structure of human hemoglobin showing the individual α- and β-molecular chains with two of the four heme groups shown as the large, open circles within rectangles. Xenobiotic protein adducts are frequently formed at the positions shown by the solid circles: the S atom of cysteine (β-chain, position 93); the amino N atom of valine (especially the N-terminal valines, α- and β-chains, position 1); the heterocyclic N atoms of histidine (most of the positions shown here); the ε-amino N atom of lysine; the N containing ring of tryptophan; the S of methionine; and the OH of tyrosine.

3 Using a Weighted Average to Calculate Exact Masses

In Figure 4 is shown a histogram of the relative abundances of the mass peak profiles at individual nominal masses that contribute to the composite mass peak profile of the adduct. Superimposed is the best fitting Gaussian distribution. While a weighted average of the same number of points on both sides of a bilaterally symmetrical Gaussian distribution estimates its exact mass, the histogram masses are slightly skewed. However, using adjacent α-chain profiles as calibrants provides nearly identical skewing for all three profiles monitored, especially when an α-chain adduct is the analyte. Consequently, very minimal error is introduced by the deviation of the profiles from a bilaterally symmetrical distribution.

4 Average Masses and Standard Deviations

In Table 1 are provided the average masses and standard deviations for triplicate measurements of the +18, +17, +16, +15, and +14 charge states for the α-chain glycate adduct and for the +17, +16, and +15 charge states of the β-chain adduct. In general, the most abundant signals provided the smallest standard deviation. The four largest signals were for the +18, +17, +16, and +15 α-chains, which provided standard deviations between 0.7 and 3.5 ppm. For future experiments, two sets of MPPSIRD data acquisitions will be made to obtain the average mass for a hemoglobin adduct; one set each for the two charge states that provide the most abundant α-chain adduct profiles. Data for two charge states ensures that adduct profiles are observed, rather than a non-adduct profile with a different charge state.

The average adduct mass errors for the +16 and +17 charge states were both -0.006 Da (0.4 ppm) with standard deviations of 0.033 Da (2.2 ppm, 0.00022%) and 0.055 Da (3.5 ppm, 0.00035%), respectively. Three of the larger standard deviations correspond to 0.165 Da. Compared to full scanning in the profile mode, SIR provides increased sensitivity, recalibration based on the lock mass each 1-sec cycle, and recalibration of the scan parameters every five cycles. Better precision and greater mass accuracy relative to the full scanning mode should result. Future experiments using full scans and the deconvolution software will provide mass accuracy and precision comparisons.

Also using a Finnigan MAT 900 mass spectrometer, Johnson et al. (4) performed peak matching experiments to determine six average masses of glycoforms of Ribonuclease B. Nine sets of 10 measurements provided average mass errors between 0.00 and 0.39 Da (<1 to 25.7 ppm). Peak matching and MPPSIRD appear to provide similar accuracy. However, MPPSIRD based on SIR descriptors with a cycle time of 1 s has the advantage that the required data can be acquired for a series of chromatographic peaks as they elute into the mass spectrometer during a single LC/ESI/MS experiment.

Fig. 2

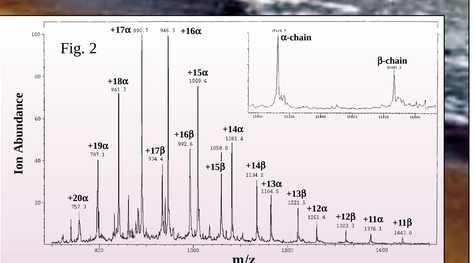

Figure 2. A 14-full-scan average of diluted, acidified human hemoglobin acquired using ESI. The deconvoluted mass spectrum appears in the inset. The charge states of the α- and β-chains of hemoglobin are labeled. The glycate adducts were observed for the blood samples studied by MPPSIRD.

Table 1. Masses of multiply charged α- and β-chain glycates measured using MPPSIRD

$[M_a+G+15H]^{+15}$	Err(ppm)[a]	M_a+G'	Err(Da)	$[M_a+G+17H]^{+17}$	Err(ppm)[a]	M_a+G'	Err(Da)	$[M_a+G+18H]^{+18}$	Err(ppm)[a]	M_a+G'	Err(Da)
Calculated 850.36860		15,288.491		Calculated 900.33099		15,288.491		Calculated 956.53868		15,288.491	
Measured 850.366	-3.1	15,288.444	-0.047	Measured 900.327	-4.4	15,288.423	-0.068	Measured 956.539	0.3	15,288.496	+0.005
Measured 850.367	-1.9	15,288.462	-0.029	Measured 900.333	2.2	15,288.525	+0.034	Measured 956.536	-2.8	15,288.448	-0.043
Measured 850.367	-1.9	15,288.462	-0.029	Measured 900.332	1.1	15,288.508	+0.017	Measured 956.540	1.4	15,288.512	+0.021
Average[d] 850.367	-2.3	15,288.456	-0.035	Average[d] 900.331	-0.4	15,288.486	-0.006	Average[d] 956.538	-0.4	15,288.486	-0.006
Std. Dev.[e] 0.001	0.7		0.010	Std. Dev.[e] 0.003	3.5		0.055	Std. Dev.[e] 0.002	2.2		0.033

$[M_a+G+15H]^{+15}$	Err(ppm)[a]	M_a+G'	Err(Da)	$[M_a+G+14H]^{+14}$	Err(ppm)[a]	M_a+G'	Err(Da)
Calculated 1020.24072		15,288.491		Calculated 1093.04306		15,288.491	
Measured 1020.245	4.2	15,288.555	+0.064	Measured 1093.107	58.5	15,289.386	+0.895
Measured 1020.246	5.2	15,288.570	+0.079	Measured 1093.047	3.6	15,288.546	+0.055
Measured 1020.247	6.2	15,288.585	+0.094	Measured 1093.106	57.6	15,289.372	+0.881
Average[d] 1020.246	5.2	15,288.570	+0.079	Average[c] 1093.087	39.9	15,289.101	+0.610
Std. Dev.[e] 0.001	1.0	0.015		Std. Dev.[e] 0.034	31.4	0.481	

$[M_a+G+17H]^{+17}$	Err(ppm)[a]	M_a+G'	Err(Da)	$[M_a+G+16H]^{+16}$	Err(ppm)[a]	M_a+G'	Err(Da)	$[M_a+G+15H]^{+15}$	Err(ppm)[a]	M_a+G'	Err(Da)
Calculated 943.91089		16,029.350		Calculated 1002.84232		16,029.350		Calculated 1069.63128		16,029.350	
Measured 943.908	-3.1	16,029.300	-0.050	Measured 1002.841	-1.3	16,029.328	-0.022	Measured 1069.623	-7.7	16,029.225	-0.125
Measured 943.876	-37.0	16,028.756	-0.594	Measured 1002.841	-1.3	16,029.328	-0.022	Measured 1069.623	-7.7	16,029.225	-0.125
Measured 943.875	-38.0	16,028.739	-0.611	Measured 1002.813	-29.2	16,028.880	-0.469	Measured 1069.626	-4.9	16,029.270	-0.080
Average[d] 943.886	-26.0	16,028.932	-0.417	Average[d] 1002.832	-10.6	16,029.179	-0.170	Average[d] 1069.624	-6.8	16,029.240	-0.109
Std. Dev.[e] 0.019	19.9	0.319		Std. Dev.[e] 0.016	16.1	0.259		Std. Dev.[e] 0.002	1.6	0.026	

(a) mass of multiply charged α- or β-chain glycate; (b) error in ppm = (measured-calculated)/calculated x 10^6; (c) mass of uncharged α- or β-chain glycate determined as $[M_a+G+nH]^{n+}$ - nH^+ where G is the glucose residue (162.14190 Da) and H weighs 1.007976 Da; (d) average of three measured values; and (e) standard deviation of three measured values.

Fig. 3

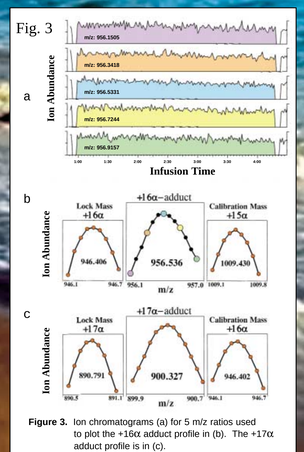

Figure 3. Ion chromatograms (a) for 5 m/z ratios used to plot the +16α adduct profile in (b). The +17α adduct profile is in (c).

Fig. 4

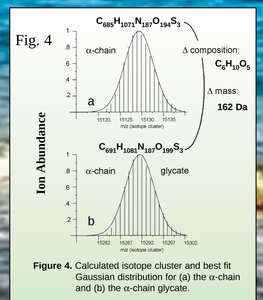

Figure 4. Calculated isotope cluster and best fit Gaussian distribution for (a) the α-chain and (b) the α-chain glycate.

5 Utility

Subtraction of the mass of the α-chain from the determined adduct mass provided 162.146 Da as the mass of the glycate residue. The calculated value based on the average masses of elements is 162.1419 Da. Twice the larger standard deviation (7.2 ppm; 0.00072%) in the α-chain mass is 0.1101 Da and dozens of ion compositions based on C_t H_t N, and O would be possible for the adduct residue within this error limit (679 ppm relative to the mass of $C_6H_{10}O_5$). Clearly, the ion composition of the adduct residue cannot be determined from this data. However, knowing an adduct's mass to within 0.2 Da can limit the number of possibilities for an unidentified adduct and provide more compelling confirmation that a particular adduct has been synthesized. In Table 2 is a list of protein post-translational modifications (from: *http://abrf.org/ABRF/ResearchCommittees/deltamass/deltamass.html).* For error limits of 1.5 to 2.3 Da, all of these modifications would be possible, but the error limit achieved using MPPSIRD (0.2 Da) limits the adduct identity to fewer possibilities – only those with ΔM of 162 Da.

Based on 980 blood samples, Espinosa et al. (5) successfully differentiated numerous animal species based on average masses of α/β-chain pairs determined with 0.02% (3 Da) mass accuracy. Of the α/β-chain pairs observed, 14% were non-diagnostic (i.e., they appeared to be present in blood from multiple species). For example, α/β-chain pairs for the horse and axis deer were 15090/16079 Da and 15091/16076 Da, respectively. The 0.2 Da mass accuracy of MPPSIRD could either confirm these mass differences, which would provide two additional diagnostically useful α/β-chain pairs, or establish that the two pairs have the same molecular weights. Some fraction of the apparently identical α/β-chain pairs based on an error limit of ±3 Da might be found to be diagnostically useful.

The three groups of amino acids in Table 3 can provide protein mass differences of from 1 to 3 Da. Hence, determination of average masses to within ±0.2 Da (0.0013%) extends upward the protein mass limit, perhaps to 40,000 Da (±0.5 Da), for which the molecular weight calculated from amino acid sequences could be confirmed unambiguously.

Table 2. Protein Post-translational Modifications

ΔM	Modification	ΔM	Modification
160	3,5-Dibromination (of Tyrosine with ^{81}Br)	160	Carboxyamidomethyl Cystenyl
161	Carboxymethyl Cystenyl	161	Methylphenylalanyl
161	Hexosamines (GalN, GlcN)		
162	N-Glucosyl (N terminus or N epsilon of Lysine) (Aminoketose)		
162	O-Glycosyl (to Serine or Threonine)		
162	Hexoses (Fru, Gal, Glc, Man)		
162	Inositol		
163	Methionyl Sulphone	163	Tyrosinyl (-Y-, -Tyr-)

Table 3. Intra-Protein Amino Acids with Mass Differences of 1 or 2 Da

Proline	97	Leucine/Isoleucine	113	Glutamine/Lysine	128
Valine	99	Asparagine	114	Glutamic acid	129
Threonine	101	Aspartic acid	115	Methionine	131
Cysteine	103				

Fig. 5

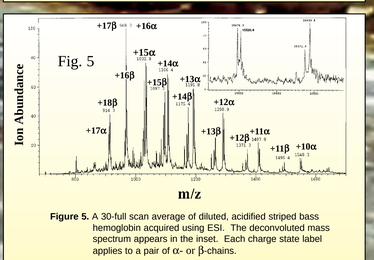

Figure 5. A 30-full scan average of diluted, acidified striped bass hemoglobin acquired using ESI. The deconvoluted mass spectrum appears in the inset. Each charge state label applies to a pair of α- or β-chains.

6 Future Experiments

We plan to utilize MPPSIRD to identify xenobiotic adducts of fish hemoglobins as a measure of environmental exposure. Human hemoglobin was employed as a model for initial studies. An average of 30 full scans for hemoglobin from a striped bass *(Roccus Saxitilis)* is shown in Figure 5. Multiple α- and β-chains are apparent.

Availability

MPPSIRD is a suite of automated procedures that enable determination of ion compositions using a double focusing mass spectrometer and an ancillary personal computer. MPPSIRD is now available from the authors for the Finnigan ICL 10.6 data system upon request.

7 CONCLUSION

Mass determinations of proteins from MPPSIRD data provide lower error limits than MALDI/TOFMS or ESI/QQQMS or ESI/ITMS measurements and thereby reduce the number of possible amino acid compositions for protein analytes. Determination of the masses of proteins and their adducts to within 0.2 Da would provide greater confidence when screening for protein adducts, for confirming that adducts have formed in laboratory experiments, for detecting mutational mass shifts, and for confirming protein sequences and syntheses.

8 REFERENCES

1. **Kassel, D.B.; Stults, J.T.; Thibault, P.** *Mass Spectrometric Characterization of Peptides & Proteins,* **ASMS Course, 1997.**
2. **Grange, A.H.; Brumley, W.C.** *A Mass Peak Profile Generation Model to Facilitate Determination of Elemental Compositions of ions Based on Exact Masses and Isotopic Abundances,* **J. Am. Soc. Mass Spectrom., 1997,** *1,* **170-182.**
3. **Grange, A.H.; Sovocool, G.W.; Donnelly, J.R.; Genicola, F.A.; Gurka, D.F.** *Identification of Pollutants in a Municipal Well Using HRMS,* **Rapid Commun. Mass Spectrom.,** *12,* **1998, 1161-1169.**
4. **Johnson, K.L.; Snyder, M.R.; Tomlinson, A.J.; Prendergast, F.G.; Naylor, S.N.** *Peak Matching Techniques for Characterizing Protein Heterogeneity,* **Proceedings of the 43rd ASMS Conference on Mass Spectrometry, 1995, 648.**
5. **Espinosa, E.O.; Lindley, N.C.; Gordon, K.M.; Ekhoff, J.A.; Kirms, M.A.** *ESIMS Analysis of Blood for Differentiation of Species,* **Analytical Biochemistry,** *268,* **1999, 252-261.**

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